OPAP IAP24
AUG 05 2010
PATENT & TRADEMARK OFFICE

DECLARATION

SIR:

I, Shunichi KURODA declare that:

1) I am one of the inventors of the above-identified application, and am familiar with the subject matter of said application as well as the disclosures in the cited references.

2) In order to demonstrate the advantage of the present invention, the following experiments were carried out under my direction and supervision.

Study-1 (Antibodies bound to BNC-ZZ)

The purpose of this Experiment was to determine the antibody species that can bound to BNC-ZZ.

Background:

BNC-ZZ is a hallow nano-sized particle, which consisted of lipid membrane and modified L-proteins embedded in the membrane. The modification of L-protein is done to its pre-S1 region to which ZZ-domain amino acid sequence (tandem Z-domains, antibody's Fc region-binding domain) of Staphylococcus aureus protein A is inserted.

The protein A is well known as an antibody binding protein. Protein A binds to Fc region of wide variety of antibody species and subclasses (Table.1). A part of protein A (Z-domain) is known to be binding domain of Fc region of antibodies (ref.1).

In this experiment we examined the binding capability of various antibodies to BNC-ZZ.

Method

BNC-ZZ (5 μg/mL of protein) was placed on the sensor plate of QCM (Quartz Crystal Microbalance) system, and then blocked with skim milk (100 μg). Various antibodies were diluted by distilled water to appropriate concentration, and each sample of 5 μL was put into the sampling pit repeatedly until no further change of frequency occurs. The total concentration of the antibody added was judged to be the total binding activity of BNC-ZZ placed on the sensor plate.

Table 1. Binding of various antibodies to Protein A

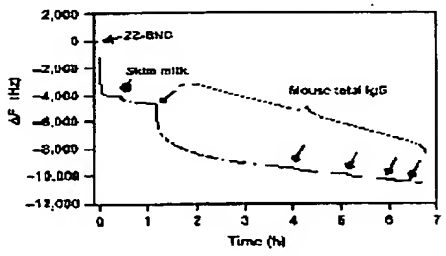
Species	Subclass	binding	Species	Subclass	binding
Human	IgA	variable	Horse		++
	IgD	–	Monkey		
	IgE	–	(rhesus)		++++
	IgG1	++++	Mouse	IgG1	+
	IgG2	++++		IgG2a	++++
	IgG3	–		IgG2b	+++
	IgG4	++++		IgG3	++
	IgM	variable		IgM	variable
Avian	IgY	–	Pig		+++
Cow		++	Rabbit	no distinction	++++
Dog		++	Rat	IgG1	–
Goat		+*+		IgG2a	–
Guinea pig	IgG1	++++		IgG2b	–
	IgG2	++++		IgG3	+
Hamster		+	Sheep		+/–

++++ = strong binding, ++ = medium binding, +/– = weak or no binding
*: binding strength of goat antibody differ depending on references

Results:

Figure 1 shows the binding profile of mouse IgG to BNC-ZZ as measured by QCM. The sensor chip was first treated with BNC-ZZ (5 μg as protein), then blocked with skim milk (100 μg), and finally reacted with excess amounts of various IgGs. As shown in the figure, BNC-ZZs of 119.4 ± 2.2 ng were bound on the sensor chip as estimated from the initial frequency change (DF) of –3979 ± 74 Hz. After repeated injection (5 times) onto the skim sensor chip, a total of 164.2 ± 7.3 ng (corresponding to DF of –5473 ± 244 Hz) (n = 3) of mouse total IgG (ca. 150 kDa) was found to be adsorbed indicating that about 0.5 mol of mouse total IgG was bound per mol of ZZ-L protein (49.1 kDa) at maximum. Assuming that only about half of the surface of BNC-ZZ bound to the sensor chip was available for IgG binding, the result suggested that each ZZ-L protein is able to bind one molecule of IgG. Because each BNC-ZZ particle contains about 130 molecules of ZZ-L protein, total of 130 molecules of IgG can binds to per one BNC-ZZ particle.

Figure 1. Binding of mouse IgG to BNC-ZZ



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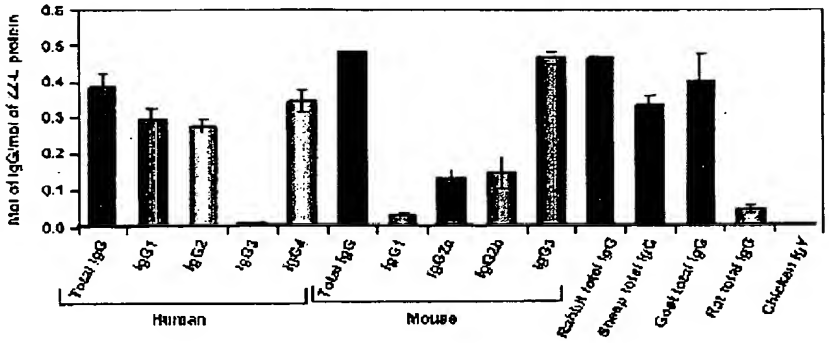
Among 16 antibodies examined, human IgG4, mouse IgG1, rat total IgG and chicken IgY did not bound to BNC-ZZ, but all other IgG species bound well to BNC-ZZ.

Figure 2. Affinity of various antibodies to BNC-ZZ



Summary and Comments:

BNC-ZZ has ability to bind to variety of IgGs across the animal species. The binding strength of IgG is similar to that reported for protein A (cf. Table 1), though there is some minor difference between the two, indicating antibodies binds to BNC-ZZ through ZZ-tag.

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Study-2 (DNA transfection by BNC-ZZ with cell specific antibodies)

The purpose of this Experiment was to demonstrate that BNC-ZZ can deliver DNA into cells using antibody specific manner.

Background:

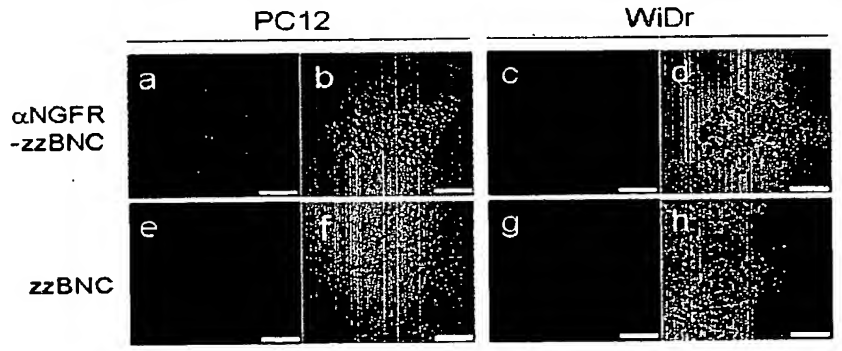
To demonstrate the antibody-specific DNA transfection of cells, three types of antibody, anti-nerve growth factor receptor (NGFR) antibody, anti-sarcoglycon (SG) antibody, and anti-CD3 antibody were used. NGFR is known to be extensively expressed in PC12 (cell line originated from rat peripheral nerve cell) and Gli36 (from human glial cell), SG in C2C12 (mouse skeletal muscle -like cell), and CD3 in Jurkat cells (from human T cell, Leukemia cell).

Methods and Results

Experiment 1: (anit-NGFR antibody-dependent pDNA transfection)

PC12 as positive cells and WiDr as control cells are used. Plasmid DNA for EGFP was encapsulated into BNC-ZZ/ liposome complex, and anti-NGFR antibody (mouse monoclonal) was mixed with the BNC-ZZ/pDNA. The mixture was then gel-filtrated with PD-10 column to remove the excess IgG. The BNC-ZZ/pDNA/IgG complex was then applied to cultured cells. After incubation for 2 days, these cells are observed by fluorescent microscopy.

Figure 3 Introduction of pDNA to PC-12 cells by anti-NGFR displaying BNC-ZZ



a,c,e,g: flurorescent microscopy b,d,f,h: phase contrast microscopy

As seen in Figure 2, green fluorescent can be seen only in PC-12 cells treated with anti-NGFR displaying

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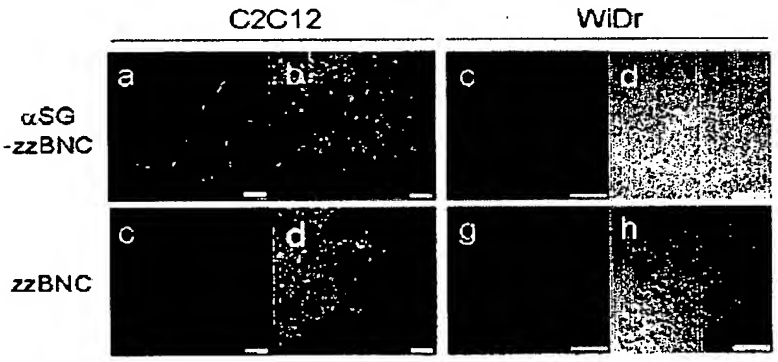
BNC-ZZ with pDNA encapsulated.. In negative control cell (WiDr) or in PC12 cells treated without antibody showed no expression of EGFP.

Experiment 2: (anti-SG antibody-dependent pDNA transfection)

C2C12 as a positive cell and A431 as a negative control cell are used. Plasmid DNA for EGFP was encapsulated into BNC-ZZ/ liposome complex, and anti-sarcoglycon (SG) antibody (mouse monoclonal) was mixed with the BNC-ZZ/pDNA. The mixture was then gel-filtrated with PD-10 column to remove the excess IgG. The BNC-ZZ/pDNA/IgG complex was then applied to cultured cells. After incubation for 2days, these cells are observed by fluorescent microscopy.

Figure 4. Introduction of pDNA to C2C12 cells by anti-sarcoglycon displaying BNC-ZZ



a,c,e,g: flurorescent microscopy, b,d,f,h: phase contrast microscopy

As seen in Figure 3, green fluorescent can be seen only in C2C12 cells treated with anti-SG displaying BNC-ZZ with pDNA encapsulated. In negative control cell (WiDr) or in C2C12 cells treated without antibody showed no expression of EGFP.

Experiment 3: (anti-CD3 antibody-dependent pDNA transfection)

Jurkat cells were used as CD3-positive cells. Plasmid DNA coding both EGFP and luciferase was encapsulated into BNC-ZZ/ liposome complex. The pDMA- encapsulated BNC-ZZ/liposome was then mixed and attached with or without anti-CD3 antibody (OKT3, monoclonal antibody, mouse IgG2a). The resulting complex was then applied to Jurkat cells. After 48 hr incubation, cells were observed for EGFP expression and determined for luciferase activity. As seen in Fig.4, Jurkat cells treated with the antibody attached complex showed a green fluorescent indicating that EGFP gene are expressed in these cells.

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As seen in Fig.5 the basal luciferase activity of non-treated cells was below 1x 10^8 and minimal. The activity in cells treated with the complex with no antibody attached was about 2.5 x 10^8. When cells were treated with antibody-displaying complex, the luciferase activity increased to 12.5 x 10^8, which is about 5-fold increase as compared with that seen in cells treated with non-antibody displaying complex.

The result indicated that BNC-ZZ can introduce pDNA into target cells with antibody-dependent manner.

Figure 5. Introduction of pDNA to Jurkat cells by anti-CD3 displaying BNC-ZZ

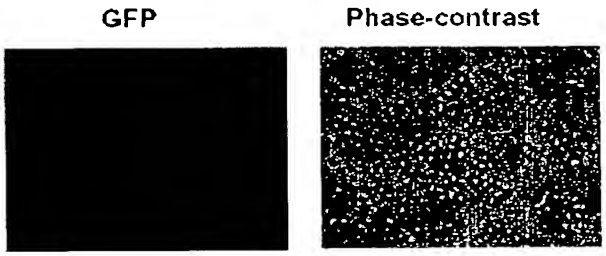
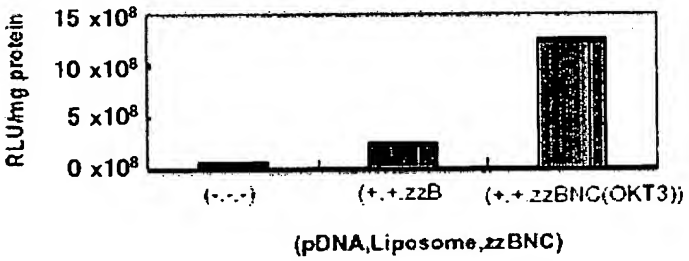


Figure 6. Luciferase activity of Jurkat cells transfected by anti-CD3 displaying BNC-ZZ



Conclusion:

In this declaration, it is clearly demonstrated that BNC-ZZ can display many antibodies though the ZZ-tag inserted in the L-protein of BNC. Using three types of antibodies, which is displayed on the surface of BNC-ZZ or of complex composed of BNC-ZZ and liposome, we have demonstrated that BNC-ZZ can introduce its encapsulated pDNA into target cells. In the document of the patent and our previous declaration, other 4 antibody species are also demonstrated to be useful for introduction of pDNA, and beads to target cells in vitro and in vivo. Taken together, BNC-ZZ is useful tool to target cells and introduce its content into the cells by using target cell-specific antibodies of various types.

Reference:

Sjödahl J. (1977):. Eur J Biochem. 78, 471-490.

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I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: July 7, 2010 Shunichi KURODA

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